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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/85, 5/10, 15/38 A61K 39/245, C12N 15/40 // C12N 15/62	A1	(11) International Publication Number: WO 94/11521 (43) International Publication Date: 26 May 1994 (26.05.94)

(21) International Application Number: PCT/CA93/00447

(22) International Filing Date: 3 November 1993 (03.11.93)

(30) Priority data:
07/975,719 10 November 1992 (10.11.92) US

(71) Applicant: BIOSTAR INC. [CA/CA]; 124 Veterinary Road, Saskatoon, Saskatchewan S7N 0W0 (CA).

(72) Inventors: KOWALSKI, Jacek ; United Biomedical Incorporated, 25 Davids Drive, Hauppaughe, NY 11788 (US). GILBERT, Scott ; Adri, P.O. Box 640, Lethbridge, Alberta T1J 3Z4 (CA). ZAMB, Timothy, J. ; 31 Brewster Lane, Strongs Neck, Setauket, NY 11733 (US).

(74) Agent: ERRATT, Judy, A.; Gowling, Strathy & Henderson, 160 Elgin Street, Suite 2600, Ottawa, Ontario K1P 1C3 (CA).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: BOVINE HEAT SHOCK PROMOTER AND USES THEREOF

(57) Abstract

A novel expression system using the heat-inducible bovine hsp70A promoter and associated cis-acting elements is disclosed. The system provides for the continuous production of a highly pure, authentic protein, substantially free of infectious viral and cellular protein contaminants.


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     TCTTCGAGAAACTCGGGAACTTTCTGTATTTTGGCTGTCCCGGCAGTCGTGTAGC   55
               HSE
     CCTTAATTCTACTTTAAACCACCAAACTAATTTGAGCCCCGAGATCCTCTCACCG   110

     CCCTACAATTAATTACAAGCCCAGGGCTGATCCTTCCAGTCGACTCGACTCCAAA   165

     CTACTTGGCTGGCTGGTCGCCAGGAAACCAGAGACAGAGTGGGTGGACCTTCCCA   220
40                                                      TTCCT-

     GCCCCTCTCCCCCTCTCCTTAGGACTCCTGTTTCCTCCAGCGAATCCTAGAAGAG   275
45   ---------------CTCA---GGGTCCCTGTCCCCTCCAGTGAATCCCAGAAGAC
                                                       HSE
     TCTGGAGAGTTCTGGGA--GGAGAGGCATCCAGGGCGCTGATTGGTTCCAGAAAG   328
83   TCTGGAGAGTTCTGAGCAGGGGGCGGCACTCTGGCCTCTGATTGGTCCAAGGAAG
         HSE                                 CCAAT
     CCAGGGGG-CAGGACTTGAGGCGAAACCCCTGGAATATTCCCGACCTGGCAGCCC   382
138  GCTGGGGGGCAGGACGGGAGGCGAAACCCCTGGAATATTCCCGACCTGGCAGCCT
                                     HSE
     CACTGAGCTCGGTCATTGGCTGACGAGGGAAAAGGCGGGGCTTGATGAAGAAT   435
193  CATCGAGCTCGGTGATTGGCTCAGAAGGGAAAAGGCGGGTCTCCGTGACGACT
                   CCAAT       Pu Box   GC
     TATAAACACAGAGCCGCCTGAGGA---GA-AACAGC-AGCCTGGAGAGAGCTGATAA   487
246  TATAAAAGCCCAGGGGCAAGCGGTCCGGATAACGGCTAGCCTGA-GG-AGCTGCTGC

     AACTTACGGCTTAGTCCGT-GAGAGCAGCTTCCGCAGACCCGCTATCTCCAAGGA   541
301  GACAGTCCACTACCTTTTTCGAGAGTGACTCCCGTTGTCCCAAGGCTTCCCAGAG

     CCGCCC---GAGG-----GGCACCAGAGCGTTCAGTTTTCGGGTTCCGAAAAGCC   588
356  CGAACCTGTGCGGCTGCAGGCACCGGCGCGTCGAGTTTCCGGCGTCCGGAAGGAC

     CGAGCTTCTCGTCGCAGATCCTCTTCACCGATTTCAGGTTTGAAGCTTATTTCGG   643
411  CGAGCTC-TTCTCGCGGATCCAGTGTTCCGTTTCCAGCCCCCAATCTCAGAGCCG

     AGCCGGAAAAG--CAGGGCACCGGCATGGCGAAAAACACAGCTATCGGCATCGAC   696
465  AGCCGACAGAGAGCAGGGAACCGC-ATGGCCAAAGCCGCGGCAGTCGGCATCGAC

     CTGGGCACCACCTACTCCTGCGTAGGGGTGTTCCAGCACGGCAAGGTGGAGATC   750
519  CTGGGCACCACCTACTCCTGCGTGGGGGTGTTCCAACACGGCAAGGTGGAGATC
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BOVINE HEAT SHOCK PROMOTER AND USES THEREOF

Technical Field

The present invention relates generally to recombinant gene expression systems. More particularly, the invention relates to novel methods for expressing and secreting gene products using the inducible bovine heat shock promoter. The invention is particularly useful for the production of pharmaceutically important polypeptides.

Background of the Invention

Proteins are conveniently produced in a variety of procaryotic and eucaryotic recombinant expression systems. These systems, however, often fail to mimic natural production such that the resulting protein lacks the authentic tertiary conformation and post-translational modifications normally present. Furthermore, expression levels are frequently inadequate, particularly in virally-vectored mammalian systems. For example, in lytic systems, expression can be severely limited by lytic functions of the virus. When high expression levels are achieved, problems with cell growth and expansion can be encountered due to the cytotoxicity of the expressed proteins. Nonlytic systems often suffer from low yields, clone instability and cytotoxicity of the final product.

Inducible expression systems have been employed in an effort to overcome some of these problems. However, most of the inducible promoters currently used in such systems are either restricted to a relatively

narrow range of host cells, are only partially inducible or are derived from organisms, such as tumor viruses, which are inherently dangerous. Accordingly, an inducible expression system which provides for the large scale synthesis of proteins, without the above-described concomitant problems, would be highly desirable.

One such candidate is a system using a promoter derived from a group of proteins known as the heat shock proteins (hsps). These proteins are ubiquitous, being found in all eucaryotic organisms studied to date, and are inducible by heat stress, as well as a variety of other external agents. Thus, cells respond to these inducers, such as elevated growth temperatures, by synthesizing high levels of hsps and coordinately reducing the rate of synthesis of other cellular proteins.

Hsps are divided into several groups on the basis of size. Of interest is the hsp70 family, so named because these proteins are approximately 70 kDa in mass. The level of synthesis of hsp70 in cells during heat shock appears to be linearly related to their thermotolerance. Li, G.C. (1985) *Int. J. Radiat. Oncol. Biol. Phys.* 11:165-177. Two human hsp70 proteins have been described -- hsp70A (Wu, B., *et al.* (1985) *Mol. Cell. Biol.* 5:330-341; Hunt, C., and Morimoto, R.I. (1985) *Proc. Natl. Acad. Sci. USA* 82:6455-6459) and hsp70B (Schiller, P., *et al.* (1988) *J. Mol. Biol.* 203:97-105). For a review of hsps, see, e.g., Morimoto *et. al.*, eds., *Stress Proteins in Biology and Medicine* (1990) Cold Spring Harbor Press; Hightower, L.E. (1991) *Cell* 66:191-197.; Craig, E.A., and Gross, C.A. (1991) *Trends Bioch. Sci.* 16:135.

The hsp70 promoter, as well as sequences in the 5′- and 3′-untranslated regions of hsp70 gene transcripts, are responsible for regulating the level of

protein and mRNA synthesis in the cell in both the induced and uninduced states (Simcox, A.A., *et al.* (1985) *Mol. Cell. Biol.* 5:3397-3402; Theodorakis, N.G., and Morimoto, R.I. (1987) *Mol. Cell. Biol.* 7:4357-4368; Yost, H.J., *et al.* (1990) in *Stress Proteins in Biology and Medicine*, Morimoto *et. al.*, eds., *Stress Proteins in Biology and Medicine* (1990) Cold Spring Harbor Press, at 379-409). A region known as the heat shock element (HSE), is found within the first 100 bp 5′ of the RNA start site of eucaryotic heat shock genes. Sorger, P.K. (1991) *Cell* 65:363. This region includes the sequence nGAAn, repeated at least two times in head-to-head or tail-to-tail orientation (nGAAnnTTCn or nTTCnnGAAn). Hsp70 genes from different species differ in the number and orientation of HSEs and in the types of other factor-binding sites found upstream. The HSE functions in stress induced promoter activation by binding a positive transactivating factor, the heat shock factor (HSF). The binding constant of this factor to the heat shock element is about a hundred fold higher than that of any other known mammalian transcription factor to its respective binding site, rendering this promoter one of the strongest.

Hsp promoters have been used to express a variety of genes. For example, Dreano, M., *et al.* (1986) *Gene* 49:1-8, describe the use of the human hsp70B promoter, as well as a *Drosophila* hsp70 promoter, to direct the heat regulated synthesis of human growth hormone, chicken lysozyme and a human influenza haemagglutinin.

EPA Publication No. 336,523 (Dreano *et al.*, published 11 October 1989) describes the *in vivo* expression of human growth hormone using a human hsp70 promoter.

PCT Publication No. WO 87/00861 (Bromley *et al.*, published 12 February 1987) describes the use of human and Drosophila hsp promoters having 5′-untranslated region variants.

EPA Publication No. 118,393 (Bromley *et al.*, published 12 September 1984) and PCT Publication No. WO 87/05935 (Bromley *et al.*, published 8 October 1987) describe the expression of *E. coli* β-galactosidase and human influenza haemagglutinin, using a *Drosophila* hsp70 promoter.

However, none of the above-described references pertains to bovine hsp promoters or to the use of these promoters to drive the expression of heterologous proteins in thermotolerant cells. Nor do any of these references describe the use of an hsp70A promoter for recombinant expression.

Disclosure of the Invention

Accordingly, the present invention provides a highly efficient inducible expression system for the production of recombinant proteins. The system allows prolonged, reversible production of proteins which mimic authentic molecules, free of potentially pathogenic agents, in large, economically useful quantities.

In one embodiment, the invention is directed to an isolated bovine hsp70 promoter capable of directing the transcription of a heterologous coding sequence positioned downstream therefrom.

In another embodiment, the subject invention is directed to an isolated bovine hsp70 5′-untranslated region.

In still another embodiment, the invention is directed to a recombinant expression construct effective in directing the transcription of a selected coding sequence. The expression construct comprises:

(a) bovine hsp70 control sequences; and

(b) a coding sequence operably linked to the control sequences, whereby the coding sequence can be transcribed and translated in a host cell, and at least one of the control sequences is heterologous to the coding sequence.

In particularly preferred embodiments, the bovine hsp70 control sequences in the expression construct comprise a nucleotide sequence substantially homologous and functionally equivalent to the sequence depicted at nucleotide positions 1 to 666, inclusive, of the upper strand of Figure 2.

Still further embodiments of the subject invention include host cells transformed with these constructs and methods of producing recombinant polypeptides using the host cells.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the following disclosure, or may be learned by practice of the invention.

Brief Description of the Figures

Figure 1 shows a map of a bovine genomic hsp70 gene λ clone and derived plasmid. Figure 1a shows the restriction map of the genomic insert in the λ EMBL3A clone. Figure 1b shows the *Bgl*II-*Xho*I fragment subcloned in pBLUESCRIPT (pBS). The region indicated by the open bar is the region sequenced and shown in figure 2. The position of the ATG initiation codon of the hsp70 gene is indicated.

Figure 2 shows a comparison of the sequence of the 5′-upstream region and a part of the coding region of the bovine hsp70A gene (numbered on the right) (SEQ ID NO:1) with the human homolog (numbered on the left) (SEQ ID NO:2). The bovine sequence corresponds to that marked by the open bar in

Figure 1b. The human hsp70A sequence corresponds to bases 40-573 of the sequence published by Hunt, C., and Morimoto, R.I. (1985) *Proc. Natl. Acad. Sci. USA* 82:6455-6459. The first 40 bp of the human sequence do not significantly match any of the bovine sequence yet determined. Transcription factor binding sites, TATAA box, and translation start codon are indicated.

Figure 3 demonstrates the heat regulated expression and secretion of bovine herpesvirus type 1 ("BHV-1") glycoproteins in transiently-transfected Madin-Darby bovine kidney ("MDBK") cells analyzed by Western blotting. Figure 3a shows an experiment with plasmid p3KHSPG3HU, expressing truncated BHV-1 glycoprotein III ("gIII"). Figure 3b shows an experiment with plasmid p3KHSPG4HU, expressing truncated BHV-1 glycoprotein IV ("gIV").

Figure 4 shows the secretion of BHV-1 gIV by a heat inducible clone (MG4-57) of stably-transformed MDBK cells over a protracted time period. Figure 4a is a depiction of a Coomassie blue-stained gel of culture medium. Figure 4b shows a quantitative ELISA determination of gIV protein in media from successive daily collections plotted cumulatively.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); *DNA Cloning*, Vols. I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* (B.D.

Hames & S.J. Higgins eds. 1984); *Animal Cell Culture* (R.K. Freshney ed. 1986); *Immobilized Cells and Enzymes* (IRL press, 1986); Perbal, B., *A Practical Guide to Molecular Cloning* (1984); the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications).

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

A "thermotolerant cell or cell line" is a cell or cell line obtained from an organism with a normal body temperature above 37°C. It has been shown that thermotolerance of cultured cells is related to the normal body temperature of the species from which they are derived. Raaphorst, G.P., *et al.* (1979) *Cancer Res.* 39:396. Generally such cells can survive and divide at temperatures above 37°C, for a number of hours and still maintain growth rates substantially the same as rates seen when the same cell is grown at 37°C.

By "bovine hsp70 promoter" is meant a DNA regulatory region derived from a bovine hsp70 gene which is capable of binding RNA polymerase and initiating transcription of a downstream (3'-direction) coding sequence. A "bovine hsp70 promoter" encompasses both promoters with identity to an hsp70 promoter isolated from a bovine species, as well as one which is substantially homologous and functionally equivalent

thereto (as defined below). The human and *Drosophila* hsp70 promoters are specifically excluded from this definition. For purposes of defining the present invention, the promoter sequence is bound at the 3′-terminus by the transcriptional start site (but does not necessarily include the site which can be provided by the 5′-UTR, described further below). The transcriptional start site is approximately 30 bps downstream (3′-direction) from the TATA box. The promoter extends upstream (5′-direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found protein binding domains (consensus sequences), responsible for binding various transcription factors and the TATA box for binding RNA polymerase. The bovine hsp70 promoter will also include one or more heat shock elements for binding heat shock factor during heat stress. A bovine hsp70A promoter sequence, isolated and cloned as described in the examples, is shown in Figure 2 and appears to include at least nucleotides 1 to 441 of the figure.

A "bovine hsp70 5′-UTR" refers to an untranslated region of nucleotides from the bovine hsp70 gene, bound at its 3′-end by the ATG codon and extending upstream (in the 5′ direction) to the hsp70 transcription start site. As explained above, this site is located approximately 30 nucleotides downstream from the TATA box.

Two DNA or polypeptide sequences are "substantially homologous" when at least about 80% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule. As used herein, substantially homologous also refers to sequences showing identity to the specified DNA or polypeptide sequence.

It is to be understood that a sequence of nucleotides or amino acids "substantially homologous" to a sequence of nucleotides or amino acids of bovine hsp70 DNA does not encompass the corresponding human or *Drosophila* hsp70 nucleotide or amino acid sequences. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., supra; *DNA Cloning*, vols I & II, supra; *Nucleic Acid Hybridization*, supra.

A sequence "functionally equivalent" to a bovine hsp70 sequence is one which functions in the same manner as the corresponding hsp70 sequence. Thus, a promoter sequence "functionally equivalent" to the bovine hsp70 promoter described herein is one which is capable of directing transcription of a downstream coding sequence above background levels.

A DNA "coding sequence" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide *in vivo* or *in vitro* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5'-(amino) terminus and a translation stop codon at the 3'-(carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) sources, viral RNA or DNA, and even synthetic nucleotide sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

DNA "control sequences" refers collectively to promoter sequences, polyadenylation signals,

transcription termination sequences, upstream regulatory domains, enhancers, and the like, untranslated regions, including 5'-UTRs and 3'-UTRs, which collectively provide for the transcription and translation of a coding sequence in a host cell.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

A control sequence "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In procaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. In eucaryotic cells, a stably transformed cell is generally one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells

through chromosome replication, or one which includes stably maintained extrachromosomal plasmids. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the exogenous DNA.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. For example, a sequence encoding a bovine protein other than an hsp is considered a heterologous sequence when linked to an hsp bovine promoter. Similarly, a sequence encoding an hsp will be considered heterologous when linked to an hsp promoter with which it is not normally associated. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Likewise, a chimeric sequence, comprising a heterologous structural gene and a gene encoding an hsp or a portion of an hsp, linked to an hsp promoter, whether derived from the same or a different hsp gene, will be considered heterologous since such chimeric constructs are not normally found in nature. Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

The term "immunogenic polypeptide" refers to a polypeptide which elicits antibodies that neutralize viral or bacterial infectivity (depending on the antigen in question), and/or mediate antibody-complement or antibody dependent cell cytotoxicity to provide protection of an immunized host. An "immunogenic polypeptide" as used herein, includes the full length (or near full length) sequence of the antigen in question, or

an immunogenic fragment thereof. By "immunogenic fragment" is meant a fragment which includes one or more epitopes and thus elicits antibodies that neutralize viral or bacterial infectivity, and/or mediate antibody-complement or antibody dependent cell cytotoxicity to provide protection of an immunized host. Such fragments will usually be at least about 5 amino acids in length, and preferably at least about 10 to 15 amino acids in length. There is no critical upper limit to the length of the fragment, which could comprise nearly the full length of the protein sequence, or even a fusion protein comprising fragments of two or more epitopes. For example, the BHV-1 gIII and gIV immunogenic polypeptides exemplified herein are fragments lacking the transmembrane binding domains of the proteins, thereby facilitating secretion of the expressed product.

B. General Methods

The present invention is based on the isolation and characterization of a bovine hsp70 promoter and the use of this promoter in an expression system for the production of heterologous proteins. The promoter is inducible. Thus, large quantities of desired proteins can be recombinantly produced by subjecting transformed cells to elevated temperatures, as well as to other known inducers of the promoter. The promoter can be used to direct the transcription of a desired protein in a wide variety of cell types. If desired, a thermotolerant cell line can be used, thereby increasing production efficiency, as well as the longevity of the host cell during recombinant production. Cis-acting control elements can be conveniently associated with the bovine hsp70 promoter in order to optimize expression of the structural gene associated therewith. These regulatory elements direct the efficient expression of the

structural gene during heat shock. If proteins produced in the system are either naturally secreted or engineered to be, the transformed cells can survive and produce the protein product for protracted time periods, further increasing yields. The system allows for the production of a desired protein in an authentic configuration, with authentic post-translation modifications, in a relatively pure form and in economically useful amounts.

The hsp70 promoter of the present invention can be isolated from a bovine genomic library using an appropriate probe and cloned for future use. Similarly, the sequence can be produced synthetically, based on the sequence depicted in Figure 2, using known methods of polynucleotide synthesis. See, e.g. Edge, M.D., *Nature* (1981) <u>292</u>:756; Nambair, *et al*. *Science* (1984) <u>223</u>:1299; Jay, Ernest, *J. Biol. Chem.* (1984) <u>259</u>:6311.

For purposes of the present invention, the bovine hsp70A promoter was isolated by screening a bovine genomic library with a human hsp70A probe, as described further below. The promoter appears to include at least the nucleotides depicted at positions 1 to 441 of Figure 2. A TATAAA box (presumed to bind transcription factor IID) is located at positions 436-441 of Figure 2. Two CCAAT boxes (the binding sites for the CCAAT box-binding transcription factor, CTF) are located at positions 314 and 397, respectively, of the figure. A purine rich element and GC element (for binding Sp1 factor) is found at position 408. Three regions including heat shock elements appear to be present at positions 3-24, 265-287 and 350-372.

The bovine hsp70 promoter, or a functional portion thereof, can be used to direct the transcription of a heterologous coding sequence when operably linked thereto. The entire promoter sequence need not be present so long as at least one heat shock element, as

well as the transcription initiation site and the RNA polymerase binding site, are present. Accordingly, a promoter can be engineered to include only these necessary sequences. Generally, for use in the present expression system, a sequence of nucleotides substantially homologous and functionally equivalent to nucleotides found at about positions 350 to 441, encompassing one heat shock element, more preferably about 265 to 441, encompassing two heat shock elements, and even nucleotides 1 to 441 and regions extending upstream from position 1 and downstream from position 441, will be used to direct the transcription of the desired heterologous coding sequence.

In order to achieve efficient expression using the bovine hsp70 promoter, it is desirable to include an hsp70 5′-UTR region in the present system. This region is bound at its 3′-end by the ATG codon and extends upstream (in the 5′ direction) to the hsp70 transcription start site. As explained above, the transcription start site is located approximately 30 nucleotides downstream from the TATA box. Thus, the bovine hsp70A 5′-UTR appears to encompass approximately 190 to 200 nucleotides upstream of the ATG codon depicted in Figure 2. This region shows approximately 65% sequence homology to the corresponding human hsp70A 5′-UTR.

The hsp70 5′-UTR region used need not be derived from a bovine host, but can be derived from another corresponding eucaryotic gene, such as from a human hsp70 gene, an insect hsp70 gene, such as from *Drosophila*, or any other eucaryotic hsp70 gene. If the bovine hsp70A promoter is used, it is preferable to use a corresponding hsp70A 5′-UTR (again, not necessarily from a bovine source). However, 5′-UTRs derived from hsp70B genes will also find use in systems utilizing the hsp70A promoter.

If an homologous 5′-UTR is utilized, it is generally provided as part of the isolated bovine hsp70 promoter and associated sequences and no further manipulation is necessary. The 5′-UTR can also be synthetically produced, based on known 5′-UTR sequences, and ligated to the hsp70 promoter sequence. Similarly, the 5′-UTR can be isolated from, or the promoter construct added to, a plasmid bearing the 5′-UTR sequence, using restriction enzymes and procedures. Site specific DNA cleavage is performed by treatment with a suitable restriction enzyme (or enzymes), under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available enzymes. See, e.g., New England Biolabs, Product Catalog. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis, using standard techniques. A general description of size separations is found in *Methods in Enzymology* (1950) 65:499-560. The 5′-UTR and promoter sequence can then be ligated to each other using known techniques.

Sequences derived from the 3′-UTR, an untranslated region flanking the 3′-end of the hsp70 structural gene, can also be used in conjunction with the present system and can be placed downstream from the coding region to increase expression efficiency thereof. The 3′-UTR appears to stabilize mRNA. As with the 5′-UTR, the 3′-UTR need not necessarily be derived from a bovine hsp gene. Rather, the 3′-UTR can come from any corresponding hsp70 gene or even the gene to be expressed, provided that the gene includes a 3′-UTR. The examples herein describe the use of a human hsp70A 3′-UTR and a *Drosophila* 3′-UTR, respectively, in combination with a bovine hsp70A promoter and 5′-UTR, to direct the

expression of a heterologous coding sequence. The 3'-UTR is ligated 3' to the desired structural gene using techniques known in the art.

Markers and amplifiers can also be employed in the subject expression systems. A variety of markers are known which are useful in selecting for transformed cell lines and generally comprise a gene whose expression confers a selectable phenotype on transformed cells when the cells are grown in an appropriate selective medium. Such markers for mammalian cell lines include, for example, the bacterial xanthine-guanine phosporibosyl transferase gene, which can be selected for in medium containing mycophenolic acid and xanthine (Mulligan *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78:2072-2076), and the aminoglycoside phosphotransferase gene (specifying a protein that inactivates the antibacterial action of neomycin/kanamycin derivatives), which can be selected for using medium containing neomycin derivatives such as G418 which are normally toxic to mammalian cells (Colbere-Garapin *et al.* (1981) *J. Mol. Biol.* 150:1-14). Useful markers for other eucaryotic expression systems, are well known to those of skill in the art.

Expression can also be amplified by placing an amplifiable gene, such as the mouse dihydrofolate reductase (dhfr) gene adjacent to the coding sequence. Cells can then be selected for methotrexate resistance in dhfr-deficient cells. See, e.g. Urlaub *et al.* (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220; Rungold *et al.* (1981) *J. Mol. and Appl. Genet.* 1:165-175.

The above-described system can be used to direct the expression of a wide variety of procaryotic, eucaryotic and viral proteins, including, for example, viral glycoproteins suitable for use as vaccine antigens, immunomodulators for regulation of the immune response, hormones, cytokines and growth factors, as well as

proteins useful in the production of other biopharmaceuticals.

The present system is particularly useful for the production of bovine viral antigens, such as, but not limited to, antigens derived from bovine herpesvirus (BHV-1), bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus, bovine rotavirus, bovine coronavirus and bovine parainfluenza virus. A number of protective antigens from these viruses are known. For example, in the case of BHV-1, the viral envelope glycoproteins gI, gIII and gIV have been isolated as well as recombinantly produced and have been shown to be effective protective antigens. (See, e.g. Babiuk, L.A., *et al.* (1987) *Virology* 159:57-66 and U.S. Patent No. 5,151,267, for a description of the isolation and cloning of these antigens, respectively). Similarly, monoclonal antibody analysis of gp53 from BVDV indicates that antibodies thereto possess virus neutralizing activity. Deregt, D., *et al.* (1990) *Can. J. Vet. Res.* 54:343-348 and the nucleotide sequence for gp53 is known (Collett, M. *et al.* (1988) *Virology* 165:191-199). Accordingly, the present invention provides a method for efficiently producing these important antigens.

The gene sequences encoding the desired protein can be isolated or obtained recombinantly, using known techniques. Alternatively, DNA sequences encoding the proteins of interest can be prepared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the particular amino acid sequence. In general, one will select preferred codons for efficient expression in the intended host. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge

(1981) *Nature* 292:756; Nambair et al. (1984) *Science* 223:1299; Jay et al. (1984) *J. Biol. Chem.* 259:6311.

It may also be desirable to produce mutants or analogs of the proteins of interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., Sambrook et al., supra; *DNA Cloning*, Vols. I and II, supra; *Nucleic Acid Hybridization*, supra.

For purposes of the present invention, it is particularly desirable to further engineer the coding sequence to effect secretion of the polypeptide from the host organism. This enhances clone stability and prevents the toxic build up of proteins in the host cell so that expression can proceed more efficiently. Homologous signal sequences can be used for this purpose with proteins normally found in association with a signal sequence. Additionally, heterologous leader sequences which provide for secretion of the protein can be added to the constructs. Preferably, processing sites will be included such that the leader fragment can be cleaved from the protein expressed therewith. (See, e.g., U.S. Patent No. 4,336,246 for a discussion of how such cleavage sites can be introduced). The leader sequence fragment typically encodes a signal peptide comprised of hydrophobic amino acids.

The choice of an appropriate leader will depend on the cell type used to express the protein. For example, sequences from genes encoding human α-interferon (Maeda et al. *Nature* (1985) 315:592), human gastrin-releasing peptide (Lebacq-Verheyden *et al.* (1988) *Molec. Cell. Biol.* 8:3129), human IL-2 (Smith *et al.* (1985)

Proc. Natl. Acad. Sci. USA 82:8404), mouse IL-3 (Miyajima *et al.* (1987) *Gene* 58:273), and human glucocerebrosidase (Martin *et al.* (1988) *DNA* 7:99), will provide for secretion of a heterologous protein in mammalian cells. These sequences can also be used to provide for secretion in insect host cells, as can DNA encoding genes for secreted insect or baculovirus proteins, such as the baculovirus gp67 gene. For expression in bacteria, DNA encoding suitable signal sequences can be derived from genes for secreted bacterial proteins, such as the *E. coli* outer membrane protein gene (*omp*A) (Ghrayeb *et al.* (1984) *EMBO J.* 3:2437) and the *E. coli* alkaline phosphatase signal sequence (*pho*A) (Oka *et al.* (1985) *Proc. Natl. Acad. Sci. USA* 82:7212). (See, also, U.S. Patent No. 4,336,336). The signal sequence of the alpha-amylase gene from various Bacillus strains can be used to secrete heterologous proteins from *B. subtilis* (Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:5582; EPO Publication No. 244,042). Finally, secretion in yeast can be directed by, i.e., the yeast invertase gene (EPO Publication No. 012,873; and the α-factor gene (U.S. Patent Nos. 4,546,083, 4,588,684 and 4,870,008).

For some proteins, particularly viral glycoproteins, secretion can be effected by deleting all or portions of the transmembrane binding domains that might be present, thereby eliminating or substantially decreasing transmembrane binding and enhancing secretion. Such an approach is described in, e.g., Motz, M., *et al.* (1987) *Gene* 58:149-154); and Spaete *et al.* (1990) *J. Virol.* 64:2922-2931.

Alternatively, molecules known to aid in the egress of the expression product from the cell can be coexpressed with the desired protein. See, e.g., Hutchinson *et al.* (1992) *J. Virol.* 66:2240-2250.

Once the coding sequences for the desired proteins have been prepared or isolated, an expression vector is constructed so that the particular coding sequence is located in the vector downstream from the bovine hsp70 promoter and the 5'-UTR, if present. Accordingly, the preferred constructs of the present invention will generally include, in order, a bovine hsp70 promoter sequence including a transcription start site, an hsp70 5'-UTR, a consensus sequence coding for translation initiation, a gene sequence coding for a desired protein engineered so that the protein will be secreted, and a 3'-UTR. Additional intervening nucleotide sequences can also be present so long as transcription and translation of the desired coding sequence is not disrupted.

The positioning and orientation of the coding sequence with respect to the control sequences is such that the coding sequence is transcribed under the direction of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the particular protein of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it can be attached to the control sequences with the appropriate orientation; i.e., to maintain the proper reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

The expression vector is then used to transform an appropriate host cell. A number of mammalian cell lines are known in the art and include immortalized cell

lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), Madin-Darby bovine kidney ("MDBK") cells, as well as others. Similarly, bacterial hosts such as *E. coli*, *Bacillus subtilis*, and *Streptococcus spp.*, will find use with the present expression constructs. Yeast hosts useful in the present invention include *inter alia*, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guillerimondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Insect cells for use with baculovirus expression vectors include, *inter alia*, *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*.

It may be desirable to use a cell line homologous to the species in which the protein is to be used, thereby assuring structural authenticity and guaranteeing a product free of heterologous interfering contaminants. Furthermore, it is particularly preferable to use a thermotolerant cell line to produce the desired protein when heat is used as the inducing agent. Such a cell can withstand prolonged elevated temperatures, allowing induction of the heat shock promoter and the concomitant production of the desired protein for an extended period of time without cell death. A number of thermotolerant cell lines are known in the art and will generally be derived from organisms that have normal body temperatures above 37°C. Thus, cells derived from bovine species (having a normal body temperature of 39°C), such as MDBK cells, will find use in the subject invention, as will cell lines derived from porcine (having a normal

body temperature of 39°C), muntjac (having a body temperature of 38.5°C), and other species.

The transformation procedure used depends upon the host to be transformed. Mammalian cells can conveniently be transformed using, for example, DEAE-dextran based procedures, calcium phosphate precipitation (Graham, F.L. and Van der Eb, A.J. (1973) *Virology* 52:456-467), protoplast fusion, liposome-mediated transfer, polybrene-mediated transfection and direct microinjection of the DNA into nuclei. Bacterial cells will generally be transformed using calcium chloride, either alone or in combination with other divalent cations and DMSO (Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989)). DNA can also be introduced into bacterial cells by electroporation. Methods of introducing exogenous DNA into yeast hosts typically include either the transformation of spheroplasts or transformation of intact yeast cells treated with alkali cations.

Proteins are then produced by growing the transformed host cells in suitable media and under conditions that will provide for expression of the same. Such conditions are known or will readily be apparent to those of skill in the art. It has been found that growth in serum-free medium, with changes every 24 hours, provides dramatically increased yields of proteins. Production of the desired protein is induced by subjecting the transformed cells to an agent known to induce the hsp promoter. Such agents include, for example, heat, metal ions, such as Cd, Zn and Cu, azetidine, forskolin, prostaglandin PGA2, adenovirus E1A protein, amino acid analogs, certain ionophores, ethanol, hydrogen peroxide and inhibitors of mitochondrial function.

A particularly preferred method of induction is the use of heat. Thus, the hsp promoter is induced by increasing the ambient temperature of the cells during growth, generally in the late stationary phase, to a temperature above 37°C. Generally, cells will be maintained at a temperature of 38 to 45°C, more preferably 39 to 44°C and most preferably 40 to 43°C, for a period of 1 to 12 hours, more preferably 4 to 6 hours, and most preferably 6 hours. Additionally, temperatures can be elevated periodically, i.e. for 1 to 10 hours a day, more preferably 3 to 6 hours a day, for a period of 1 to 21 days or more. Other suitable temperatures and time periods can be readily determined by one of skill in the art to assure the efficiency of the hsp70 promoter, thereby maximizing production levels of the desired product.

The protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the protein can be used directly or purified from the media. If the protein is not secreted, it is isolated from cell lysates. The selection of an appropriate recovery method is within the skill of the art.

The constructs can also be used in gene therapy or nucleic acid immunization, to direct the production of the desired gene product *in vivo*, by administering the expression constructs directly to a subject for the *in vivo* translation thereof. See, e.g. EPA Publication No. 336,523 (Dreano *et al.*, published 11 October 1989). Alternatively, gene transfer can be accomplished by transfecting the subject's cells or tissues with the expression constructs *ex vivo* and reintroducing the transformed material into the host. The constructs can be directly introduced into the host organism, i.e., by injection (see International Publication No. WO/90/11092;

and Wolff *et al.*, (1990) *Science* 247:1465-1468). Liposome-mediated gene transfer can also be accomplished using known methods. *See, e.g.*, Hazinski *et al.*, (1991) *Am. J. Respir. Cell Mol. Biol.* 4:206-209; Brigham *et al.* (1989) *Am. J. Med. Sci.* 298:278-281; Canonico *et al.* (1991) *Clin. Res.* 39:219A; and Nabel *et al.* (1990) *Science* 249:1285-1288. Targeting agents, such as antibodies directed against surface antigens expressed on specific cell types, can be covalently conjugated to the liposomal surface so that the nucleic acid can be delivered to specific tissues and cells for local administration. Following introduction of the expression constructs into the host organism, the animals can be heat treated to stimulate production of the desired protein using, i.e., a ventilated incubator, as described in EPA Publication No. 336,523 (Dreano *et al.*, published 11 October 1989). Alternatively, animals can be exposed to fever inducing agents or other stressors, in order to induce the hsp promoter.

C. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Materials and Methods

Enzymes were purchased from commercial sources, and used according to the manufacturers' directions.

Radionucleotides and nitrocellulose filters were also purchased from commercial sources.

In the cloning of DNA fragments, except where noted, all DNA manipulations were done according to standard procedures. See Sambrook et al., supra. Restriction enzymes, T_4 DNA ligase, *E. coli* DNA polymerase I, Klenow fragment, and other biological reagents were purchased from commercial suppliers and used according to the manufacturers' directions. Double stranded DNA fragments were separated on agarose gels.

Cells and DNA Transfections

Madin-Darby bovine kidney (MDBK) cells (ATCC Accession No. CCL22) were propagated in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Transient DEAE-dextran-mediated DNA transfections were performed as described by Kriegler (Kriegler, M., (1990) *Gene Transfer and Expression* (Stockton Press)). Stable transfections were performed using 5 μg of DNA and 40 μg of Lipofectin (Felgner, P.L., *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:7413-7417) per 4 x 10^6 cells. Neomycin-resistant clones (about 20/μg DNA) were selected by growth in the presence of 666 μg/ml G418 (Fehler, F., *et al.* (1992) *J. Virol.* 66:831-839, and maintained in 400 μg/ml.

Monoclonal Antibody Analysis of Secreted gIV

Indirect ELISA's were used to determine the yields of gIV as previously described (van Drunen-Littel-van den Hurk, S., *et al.*, supra). The antigenic properties of truncated gIV secreted by MDBK cells were assessed in an indirect ELISA assay using media containing equivalent amounts of gIV from transfected MDBK cells and recombinant vaccinia virus infected BSC-1 cells, serially diluted and adsorbed to plates. The

reactivity of vaccinia-produced and BHV-1-produced full-length gIV has been previously compared and found to be identical (van Drunen-Littel-van den Hurk, *et al. Vaccine* (In Press)). Individual or mixed gIV-specific monoclonal antibodies, followed by horseradish peroxidase-conjugated goat anti-mouse IgG were used for detection as previously described (van Drunen-Littel-van den Hurk, S., *et al.* (1984), supra; Hughes, G., *et al.*, supra).

Example 1
Cloning and Identification of the Bovine hsp70 Promoter

A total of 3 x 10^6 plaques of a bovine genomic library in λ EMBL3A (Frischauf, A.M. *et al.* (1983) *J. Mol. Biol.* 170:827) were screened at high stringency with a probe produced from a human hsp70 cDNA clone, pH2.3 (Wu, B., *et al.* (1985) *Mol. Cell. Biol.* 5:330-341). Five positive clones were selected and amplified. Three of these clones were unstable, since the λ phage was lost upon passage. The two remaining clones were identical and therefore likely to be clonally related. One of these was selected for further analysis. The restriction map of the genomic insert in this clone is shown in Figure 1a. Southern blot analysis using a fragment of the human hsp70 cDNA clone was used to identify a fragment of the bovine genome clone with homology to the 5′ end of the human hsp70 mRNA. This *Bgl*II-*Xho*I fragment was subcloned into pBS KSII+ (Stratagene; Alting-Mees, M.A., and Short, J.M. (1989) *Nucleic Acids Res.* 17:9494) for more detailed restriction enzyme analysis and sequencing.

DNA inserts in pBS KSII+ were sequenced as denatured double-stranded templates using a T7 sequencing kit (Pharmacia) and ^{35}S-dATP (Amersham). Reaction products were analyzed by standard procedures. Data were

analyzed and compared to GenBank files with sequence analysis software (IBI, Intelligenetics Inc.). All DNA sequences were determined by reading the template at least once in each direction.

The entire 1315 bp *Eco*RI-*Xho*I fragment shown between positions 2000 and 3300 in Figure 1b was sequenced using this procedure. The sequence of the last 750 bp is shown in Figure 2 where it is compared to the corresponding region of the human hsp70A gene. The sequence from positions 667-750 constitutes a partial open reading frame. Translation of the sequence revealed that 25 of the 28 encoded amino acids are identical to the first 28 amino acids of human hsp70A protein (Hunt, C., and Morimoto, R.I. (1985) *Proc. Natl. Acad. Sci. USA* 82:6455-6459). The TATAAA box (presumed transcription factor IID binding site) is located at position 436. The human and bovine promoter appear to share approximately 70% homology over the -79 and +20 positions. The 5'-UTR of the bovine mRNA is approximately 200 nucleotides long versus 215 for the human hsp70 mRNA and shares 60% homology with the human RNA of this region. The general organization of the promoter is similar to the human hsp70A promoter (Williams, G.T. and Morimoto, R.I. (1990) *Mol. Cell. Biol.* 10:3125; Abravaya, *et al.* (1991) *Mol. Cell Biol.* 11:586-592). The relative placement of the heat shock elements (heat shock factor binding sites, HSE) CCAAT box [CCAAT-box binding transcription factor (CTF) binding site], purine-rich element, and GC element (Sp1 factor binding site) between positions 315 and the TATAAA element is the same as the placement of these regions in the human promoter, with 86% sequence identity over this region.

The consensus heat shock element is currently defined as three or more perfect and imperfect repeats of the sequence NGAAN in a head-to-head or head-to-tail

orientation (Lis, J.T., *et al.*, (1990) in *Stress Proteins in Biology and Medicine*, Morimoto, R.I., *et al.*, eds. (Cold Spring Harbor Press)). The bovine hsp70 upstream region has three sites that meet these criteria. Two occur at position 265-287 and at position 350-372 where the bovine and human sequences are almost identical. The NGAAN repeats in the latter of these two have been shown to be protected in *in vivo* footprinting experiments (Abravaya, K., *et al.*, supra). A second cluster of NGAAN elements is found in the bovine sequence at position 3-24 in the region for which there is no corresponding human sequence available. Three of the 5 NGAAN-type repeats in this region have the correct spacing with respect to each other for a consensus heat shock element (position 12-24).

These results show that the promoter region of the bovine homolog of the human hsp70A gene has been isolated and sequenced. This gene is similar to the human hsp70A gene. The first 28 amino acids of the bovine hsp protein show 90% identity with that of the human hsp70 protein. The promoter region shows conservation of recognizable transcription factor binding sites and their spacing relative to one another (Figure 2).

Example 2
Expression Plasmid Construction For BHV-1 gIII and gIV

The ability of the bovine hsp70A heat shock promoter to direct the expression of a heterologous protein in a construct containing the bovine hsp70A 5'-UTR and a human hsp70A 3'-UTR, was tested using sequences encoding secreted forms of two BHV-1 glycoproteins as follows. After locating the translation initiation codon of the hsp70 open reading frame by sequencing, primers were synthesized for a polymerase chain reaction (PCR).

The PCR produced a 530 bp promoter fragment starting with the *Sal*I site shown in Figure 1b and terminating with an *Nco*I site that incorporates the ATG initiation codon of the hsp70A gene. Plasmids with longer upstream regions were made by rejoining this fragment to upstream hsp70 fragments via the *Sal*I site.

The BHV-1 gIII gene coding for a secreted form of the protein was obtained from a plasmid where a linker with stop codons in all three reading frames and an *Spe*I site had been inserted into the *Spl*I site immediately upstream of the transmembrane anchor, terminating the protein at amino acid 465 (Fitzpatrick, D., *et al.* (1989) *Virology* 173:46-57). A human hsp70A 3'-UTR fragment with *Spe*I and *Cla*I termini was obtained from plasmid pH2.3 (Wu, B., *et al.* (1985) *Mol. Cell. Biol.* 5:330-341) by PCR. This was placed behind the truncated gIII coding sequence creating plasmid p3KHSPG3HU. This plasmid contained 3 kb of bovine hsp70 upstream sequence, including a bovine hsp70 5'-UTR, with the BHV-1 protein start codons in precisely the same location as that of the bovine hsp70 protein.

The DNA fragment coding for a secreted form of BHV-1 gIV was obtained by modifying the 5'-end to an *Nco*I site at the start codon and by inserting a 3-frame stop codon linker at the *Sac*II site immediately upstream of the transmembrane anchor terminating the protein at amino acid 320 (Tikoo, T.K., *et al.* (1990) *J. Virol.* 64:5132-5142). This fragment was used to replace the gIII sequences in p3KHSPG3HU, creating p3KHSPG4HU.

Plasmids for the generation of stably-transfected cell lines were constructed by inserting the cassette for the expression of aminoglycoside phosphotransferase gene from pSV2NEO (Southern, P.J., and Berg, P., (1982) *Molecular and Applied Genetics* 1:327-341) into the constructs described above, immediately

behind the human hsp70 3'-UTR, yielding plasmids pG3HUNEO and pG4HUNEO (ATCC Accession Nos. 69075 and 69076, respectively), including the coding sequences for truncated gIII and gIV, respectively.

The DNA backbone for all these constructs was provided by the plasmid pPOL26 (George, H.J., et al. (1987) *Biotechnology* 5:600-603).

Example 3

Expression of BHV-1 gIII and gIV using Plasmids p3KHSPG3HU and p3KHSPG4HU

Transient assays for expression and secretion of the BHV-1 glycoproteins from plasmids p3KHSPG3HU and p3KHSPG4HU, harboring the gIII and gIV genes, respectively, driven by the bovine hsp70A promoter and 5'-UTR, and a human hsp70A 3'-UTR, were conducted as follows.

MDBK cells were transformed with the above expression constructs using the DEAE-dextran method as described in Materials and Methods. Transiently transfected cell cultures were washed twice to remove serum and incubated at either 37°C or 43°C in a minimal volume (5 ml/75 cm^2) of serum-free MEM or OptiMEM I (Gibco). At the end of the incubation period the medium was collected and centrifuged for 5 min at 2000 x g to remove cells and debris. Medium was dialyzed and lyophilized to dryness. Samples were denatured, resolved by electrophoresis in 7.5% Miniprotean gels (Bio-Rad), and proteins detected by Western blotting. The primary antibody was a 1:2000 dilution of a pool of monoclonal antibodies against either BHV-1 gIII or gIV proteins (van Drunen-Littel-van den Hurk, S., *et al.* (1984) *Virology* 135:466-479; Hughes, G., *et al.* (1988) *Archives of Virology* 103:47-60). The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG.

The results of transient assays for expression and secretion of BHV-1 glycoproteins in MDBK cells are shown in Figure 3. Figure 3a shows expression of truncated gIII from plasmid p3KHSPG3HU. Lanes 1-5 represent analysis of cell culture media added to washed cells at the start of the time interval (described below). Lanes 6-9 represent analysis of 10% of cell extracts. Lanes 1 and 6 show untransfected cells; lanes 2 and 7: 37°C, 3 h; Lanes 3 and 8: 43°C, 3 h; lane 4: 43°C, 3 h, after which cells were washed and subjected to 37°C, 2 h; lanes 5 and 9: 43°C, 6 h. The four prominent marker bands (nonnumbered lanes) correspond to 116, 97, 66 and 45 kDa biotinylated molecular weight markers detected with avidin-horseradish peroxidase. Basal level synthesis was barely detectable (lane 2) but up-shift to 43°C produced a significant amount of gIII in the medium (lane 3). There was more gIII protein in the medium after 6 h at 43°C (lane 5) than after 3 h, indicating that the process of secretion continued beyond 3 h at 43°C. Secretion appeared to be efficient, since cellular extracts examined in lanes 6-9 showed no evidence of intracellular accumulation of gIII.

The expression of truncated gIV from p3KHSPG4HU is shown in Figure 3b. Lane 1 shows purified truncated gIV protein synthesized and secreted in a vaccinia virus expression system. Lanes 2-5 show MDBK cells transfected with p3KHSPG4HU. Cell culture media was assayed 24 h later. Cells were washed and incubated for 2 h at 37°C (lanes 2 and 4) or 43°C (lanes 3 and 5) in medium without (lanes 2 and 3) or with (lanes 4 and 5) 2.5 μg/ml Brefeldin A. Cells were preincubated for 1 h in the drug prior to incubation at 37°C or 43°C. The rate of incorporation of ^{35}S-methionine into acid-precipitable material was unaffected by concentrations of Brefeldin A as high as 8 μg/ml. The four prominent marker bands

(nonnumbered lanes) correspond to 116, 97, 66 and 45 kDa biotinylated molecular weight markers detected with avidin-horseradish peroxidase. Again the basal level of synthesis was barely detectable (lane 2). An up-shift to 43°C for 3 h caused a dramatic increase in the amount of the protein in the medium (lane 3). Lanes 4 and 5 show that the presence of gIV in the medium is actually the result of secretion and not the detachment and lysis of cells since Brefeldin A, a specific inhibitor of transport between the endoplasmic reticulum and Golgi apparatus, blocks this process (lanes 4 and 5). The band that appears immediately above gIV in lanes 2-5 is residual bovine serum albumin that is not removed by washing the cells.

Example 4

Protracted Regulated Expression and Secretion of BHV-1gIII and gIV from MDBK Cells Stably Transfected with Plasmids pG3HUNEO and pG4HUNEO

The expression and secretion of gIII and gIV from plasmids pG3HUNEO and pG4HUNEO (ATCC Accession Nos. 69075 and 69076, respectively), from Example 2, was tested as follows. These plasmids include the coding sequences for truncated gIII and gIV, respectively, driven by the bovine hsp70A promoter and 5'-UTR, a human hsp70A 3'-UTR and a cassette coding for aminoglycoside phosphotransferase.

MDBK cells were transfected and treated as described above to generate stable cell lines resistant to G418. Equal numbers of drug-resistant clones were obtained with each construct. After incubation at either 37°C or 43°, as described, medium from the stably transfected clones was concentrated 4-fold for gel analysis (figure 4a) or left undiluted (figure 4b, table 1). A dot-immunoblot assay was conducted on the medium.

Of 160 neomycin-resistant clones tested, 100 were found to be heat inducible for glycoprotein synthesis and secretion. The remainder were negative at both temperatures. Clones varied in the amount of glycoprotein produced as well as in the ratio between basal and induced protein levels. No differences were observed between the distribution of expression phenotypes for gIII- and gIV-producing clones. The induction properties of 7 gIV-producing clones were followed through 80 cell doublings with no noticeable change.

One gIV clone, designated MG4-57, was tested for synthesis and secretion of glycoprotein over a protracted time period. Clone MG4-57 was grown to confluence in 4% serum containing culture medium in a 150 cm^2 flask (4×10^7 cells) and the flask was incubated with 10 ml serum-free medium at 43°C for 6 h every day for 8 days denoting 8 cycles. This medium was tested for yield of gIV as shown in Figure 4. The culture produced gIV in a quantity of 75 $\mu g/150cm^2$ (4×10^7 cells) for each 6 h period at 43°C and the ability to synthesize and secrete gIV did not decrease even after 8 cycles. Qualitatively similar results were observed in a parallel experiment with a gIII-secreting clone. Subsequent experiments indicate that lowering the temperature back to 37°C does not result in an immediate cessation of synthesis of gIV by the MG4-57 cell line. Consequently, best yields are obtained by performing a 6 h, 43°C heat shock once in every 24 h, keeping the cells in serum-free medium throughout, and changing the medium once every 24 h (immediately prior to heat shock). This medium contains gIV at a level of 10-15 $\mu g/ml$ and the experiment has been extended as far as 21 days. Total yield is thus 21 days X 13 mls X 10-15 $\mu g/ml$ = 3-4 mg per 150 cm^2 flask, or 4 X 10^7 cells.

These results and those in Example 3 show that the bovine hsp70A gene is functional in the heat regulated expression of recombinant BHV-1 glycoproteins and thus is not a bovine heat shock cognate or pseudogene. The results also show that the constructs respond, in transient transfections, to both increases and decreases in temperature. Stably-transfected cell lines made with these constructs show a high proportion of clones with heat regulatable expression.

Example 5

Antigenic Authenticity of gIV Produced by Expression Plasmid pG4HUNEO

The truncated gIV secreted by stably-transfected MDBK cells described in Example 4 was reacted with a panel of monoclonal antibodies directed against both continuous and discontinuous epitopes of full length gIV (van Drunen-Little-van den Hurk, S., *et al.* (1984), supra; Hughes, G., *et al.* (1988), supra). The results are shown in Table 1.

Table 1. Reactivity of Monoclonal Antibodies with Full-Length and Truncated gIV.

MAb[a]	Epitope Specificity[b]	Neutralizing Activity[c]	ELISA Titer[d] VVgIV	VVΔIV	hspΔIV
135	Ia	++	640	1280	1280
9D6	Ib	+	640	640	1280
3E7	II	+	160	160	160
10C2	IIIa	++	320	320	160
4C1	IIIb	+	320	320	160
2C8	IIIc	++	160	40	80
3C1	IIId	++	80	80	80
3DpS	IV	-	1280	640	320

[a] Monoclonal antibodies developed by Hughes, G., *et al.*(1988) *Archives of Virology* 103:47-60.

[b] gIV epitopes assigned by competitive binding assays (Hughes, *et al.*, supra).

[c] Neutralizing antibody titers determined for ascites fluids. - titer: <100; + titer: >100; ++ titer: >10,000 (Hughes, *et al.*, supra).

[d] Antigen titer was expressed as the reciprocal of the highest dilution of glycoprotein IV giving a reading of at least 0.05 O.D. (492 nm). A 1:10 dilution corresponds to 0.04 μg of glycoprotein IV per well. VVgIV = affinity purified full-length gIV produced in vaccinia virus-infected BSC-1 cells and suspended in OptiMEM I medium (GIBCO) (van Drunen Littel-van den Hurk *et al.* *Vaccine*, in press). VVΔIV = affinity-purified truncated gIV produced in vaccinia virus-infected BSC-1 cells and suspended in OptiMEM. hspΔIV = truncated gIV, expressed under Hsp70A promoter and secreted in OptiMEM by transfected MDBK cells.

The reactivity was not significantly different from that of truncated and full-length gIV produced in a vaccinia virus expression system. The latter two proteins have been compared to authentic full-length BHV-1 gIV, field-tested in cattle, and found to be highly protective against BHV-1 infection (Van Drunen-Littel-van den Hurk, S., et al., *Vaccine*, In Press).

Several reports suggest that BHV-1 gIV has cytotoxic properties based on observations that it is very difficult to isolate stably-transfected cell lines expressing the native form of this protein (Fehler, F., *et al*. (1992) *J. Virol*. 66:831-839). Even though the hsp70A promoter shows some basal activity at 37°C (see Example 3), the cytotoxicity of the product was overcome by using a truncated secreted form of the protein. No difference was observed in the number of stable clones and their inducible expression properties between constructs expressing gIII and gIV. While not wishing to be bound by theory, it is postulated that the cytotoxicity of gIV is either dependent on its location in the membrane or on the maintenance of a configuration that is lost in the truncated form. However, as demonstrated herein, the reactivity of the protein with several monoclonal antibodies is not substantially altered, suggesting that it is an effective vaccine immunogen.

In summary, the expression system has several important practical advantages for the production of vaccines. A single 150 cm^2 flask of 4 x 10^7 stationary-phase cells can produce approximately 3 to 4 mg of antigen (about 250 doses) with minimal manipulations involving temperature shifts and media collection. Production is still linear after 8 temperature-shift cycles, so that extension and optimization of the protocol will likely improve yields. This result also

demonstrates that the transfected cells can be maintained in stationary phase, for protracted periods, while fully retaining the capacity to produce the recombinant protein. In comparison, a vaccinia/BSC-1 system based on a consensus strong late viral promoter, which is one of the most productive mammalian-based expression systems available, produced only twice this amount of secreted gIV (van Drunen-Little-van den Hurk, *in press*, supra).

Example 6

Construction of an Alternative BHV-1 Expression Plasmid Utilizing a *Drosophila* 3'-UTR

An expression plasmid was constructed as above, using the truncated BHV-1 gIII gene and a *Drosophila melanogastor* hsp70 3'-UTR, derived from p1730R (Voellmy, R., *et al.* (1985) *Proc. Natl. Acad. Sci. USA* 82:4949-4953) in place of the human 3'-UTR. The resulting plasmid, pGIIIA'DU, comprising the bovine hsp70A promoter and 5'-UTR, the truncated gIII gene and the *D. melanogastor* 3'-UTR, was transfected into MDBK cells as described in Example 3. The cultures were heat shocked at 24 hrs post-transfection and media and cell extracts analyzed by Western blotting, as described in Example 3.

All of the detectable gIII was found in the medium, indicating that the protein was indeed expressed and secreted. A low basal level of synthesis of gIII at 37°C was also seen. Shifting the cells to 43°C resulted in a large induction of synthesis and secretion, presumably due to the efficient induction of transcription and translation driven by the bovine hsp70A promoter and associated 5'-UTR. Secretion proceeded efficiently at 43°C for at least 3 to 6 hrs. This experiment shows that the bovine hsp70A promoter and 5'-UTR, along with the *D. melanogastor* 3'-UTR, effectively

direct regulated expression of a BHV-1 glycoprotein in transformed cells.

Example 7

Heat Regulated Synthesis of *E. coli* β-galactosidase

The ability of the bovine hsp70A promoter to express a procaryotic protein was tested using the *E. coli* Lac Z gene. Plasmids were constructed by cloning the *Sal*I-*Nco*I fragment that incorporates the ATG initiation codon of the hsp70A gene shown in Figure 1b, as described above, into plasmid pPOL26 (George, H.J., et al., (1987) *Biotechnology* 5:600-603) containing the Lac Z sequence. The coding sequence was immediately followed by a translation stop codon and the SV40 small t intron and polyadenylation signal. This construct was introduced into MDBK cells by DEAE dextran-mediated DNA transfection, as described above. β-galactosidase production was monitored histochemically by the addition of X-gal to fixed cells and by assay of β-galactosidase activity in cell extracts. The experiments showed that the bovine hsp70 promoter directed the production of amounts of β-galactosidase twenty-fold higher than those produced by plasmids utilizing the SV40 early promoter for the same purpose.

Example 8

Expression of BVDV gp53 Using the Hsp70 Promoter

BVDV is a positive stranded RNA virus. Accordingly, the gp53 gene was cloned by reverse transcription of RNA prepared from infected cells, followed by a polymerase chain reaction to amplify the DNA. PCR primers were designed so as to provide an *Nco*I site incorporating a start codon in front of amino acid 666 of the NADL strain sequence, and a stop codon and a

SalI site after amino acid 1036. This DNA was inserted into pG4HUNEO in place of the BHV-1 gIV coding sequence. The resulting plasmid, designated pGP53HUNEO was transfected into bovine MDBK cells and produced a truncated gp53 (tgp53), as described above.

Production of tgp53 in stably transfected MDBK cells was induced by increasing the incubation temperature to 43°C for 6 hrs, after which time the cells were incubated at 37°C for 2 to 4 days. Following incubation, cell culture media was collected and centrifuged for 5 min at 1500 x g to remove cells and debris. Secreted tgp53 was purified from the supernatant by immunoaffinity chromatography using a column of monoclonal antibodies to gp53 linked to Affi-Gel 10™ (Biorad) (van den Hurk, J.V. and van Drunen Littel-van den Hurk, S., (1992) *Arch. Virol.* <u>126</u>:195-213). The concentrations of tgp53 were determined by ELISA.

Two rabbits were immunized twice with 10 μg of affinity purified tgp53. Both animals developed virus neutralizing antibodies against a wide variety of BVDV strains, as shown in Table 2.

Table 2. Virus-neutralizing titers of rabbit 23 immunized with affinity purified tgp53 from MDBK cells to BVDV isolates.

BVDV isolate	Characteristics isolate	VN titer
C1	Bovine, mucosal disease, NCP, Saskatchewan	2,500
C3	Bovine, mucosal disease, CP, Saskatchewan	2,500
126	Bovine, weak neonatal calf, NCP, Nebraska	5,000
177	Bovine, aborted fetus, NCP, Nebraska	2,500
296	Bovine, mucosal disease, CP, Nebraska	2,500
365	Bovine, weak calf, CP, Nebraska	2,500
403	Bovine, aborted fetus, NCP, Nebraska	2,500
415	Ovine, acute respiratory disease, NCP, Nebraska	2,500
804	Ovine, acute respiratory disease, NCP, Nebraska	2,500
998	Bovine, mucosal disease, CP, Nebraska	2,500
CD87	Bovine acute disease, thrombocytopenia, New York	125
JV73	Bovine, mucosal disease, CP, Saskatchewan	5,000
JV580	Bovine, mucosal disease, CP, Saskatchewan	2,500
SB890	Bovine acute disease, thrombocytopenia, NCP, Iowa	250
CL1494	Bovine, mucosal disease, CP, Saskatchewan	250
Waters	Bovine, persistently infected, NCP, Saskatchewan	2,500
Draper	Bovine, laboratory strain, NCP	2,500
Oregon	Bovine, laboratory strain, CP	10,000
NADL	Bovine, laboratory strain, CP	10,000
NY-1	Bovine, laboratory strain, NCP	2,500
Singer	Bovine, laboratory strain, CP	5,000

CP: cytopathic BVDV; NCP: noncytopathic BVDV

Example 9

Expression of Follistatin Using the Hsp70 Promoter

Biologically active porcine follistatin was produced using the bovine heat shock promotor as follows. Transfer plasmid pGEM-FS was constructed from RNA isolated from 2 g ovarian tissue obtained from a sow in luteal phase of the estrus cycle. PCR primers were designed to amplify the follistatin cDNA sequence and the reverse transcriptase-polymerase chain reaction products were cloned into pGEM 3Zf(-)(Promega, Nepean, Ontario). Transformation of the *E. coli* strain JM109 (Stratagene, La Jolla, CA) produced pGEM-FS. The identity of the cloned plasmid was confirmed by restriction endonuclease mapping and single strand DNA sequencing.

The pGEM-FS containing the follistatin gene was digested with NcoI-BamHI and ligated into the heat shock expression plasmid pG4HUNEO (Example 2), replacing the BHV-1 gIV coding sequence to render pFSHUNEO. MDBK cells were transformed with pFSHUNEO using the DEAE-dextran method described above. Neomycin resistant clones were selected by growth in the presence of 666 μg/ml G418 (a neomycin analog). Stable cell lines were selected following two rounds of single cell cloning.

The transformed MDBK cells were subjected to 6 hours heat shock at 43°C followed by 18 hours culture at 37°C. The supernatants were harvested and stored frozen until use. For immunological activity assays, 250μl of media from heat shocked pFSHUNEO transformed cells was concentrated 40 times, and subjected to SDS PAGE on 10% Laemmli gels and Western blots prepared. The blots were probed with rabbit anti-porcine follistatin (obtained from Dr. Ling, Whittier Institute, La Jolla, CA) and antibody binding visualized using peroxidase linked secondary antibodies and 4-chloronapthol colormetric reagents. The blots confirmed that the recombinant

follistatin was reactive with the rabbit antisera to porcine follistatin.

Biological activity was assessed using an *in vitro* anterior pituitary cell culture system. Briefly, anterior pituitary tissues were removed from female Sprague-Dawley rats in proestrus as determined by vaginal lavage. The pituitary tissue was cut to 1-2 mm pieces, suspended in calcium and magnesium-free Hanks (CMFH) solution containing 18500 units collagenase (Type 11 Sigma), 500 units deoxyribonuclease (DNase 1 Sigma) and 0.1% bovine serum albumin (BSA Sigma) and incubated for 1.5 hours at 37°C with constant agitation. After 1 hour incubation, the tissue was dispersed by drawing approximately twenty times through a sterile, siliconized pasteur pipette. After a further 30 minute incubation, the tissue suspension was centrifuged at 200 x g for eight minutes. The supernatant was decanted and tissues further incubated for twenty minutes in CMFH containing 1X pancreatin (pancreatin 4XNF Gibco), 500 units DNase 1 and 0.1% BSA. The remaining tissue was resuspended and centrifuged as described above. The supernatant was decanted and 1 ml MEM containing 0.1% BSA, 500 units DNase 1 and 1 ml apoprotinin (Sigma) was added to the cell pellet. The pellet was immediately resuspended, incubated at room temperature for two minutes after which time 10 ml MEM plus 0.1% BSA was added. The solution was centrifuged as described above, washed twice, and viable cells enumerated. 6×10^5 cells were plated into an MEM supplemented with 10% FBS containing 60μl of a 1:4 dilution of Matrigel (Collaborative Biomedical Products, Bedford, MA) and incubated overnight. The cell media was replaced with MEM containing no FBS. After six hours, 10 nM GnRH (Sigma, St. Louis, MO) was added to each well. 1-1/2 hours later, 300 μl of supernatant from heat shocked pFSHUNEO transfected MDBK cells was added to each

well. Cell cultures were incubated overnight, after which time the supernatants were removed and stored frozen until assayed for FSH activity. The FSH radio immunoassay kit was obtained from the National Institutes of Health. The results of this assay indicated that the recombinant FS was biologically active and suppressed FSH secretion by pituitary cells.

Thus, a novel bovine hsp70 promoter and the use of the promoter in efficient expression systems have been described. Although preferred embodiments of the subject invention have been described in some detail, it is to be understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

Deposits of Strains Useful in Practicing the Invention

A deposit of biologically pure cultures of the following strains was made with the international depository authority, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, pursuant to the terms of the Budapest Treaty. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. Access to said cultures and information pertaining thereto will only be given to those authorities, natural persons or legal entities entitled therto under Rule 11 of the Budapest Treaty and subject to the conditions as provided in that Rule. The designated deposits will be maintained for a period of at least thirty (30) years from the date of deposit, or for five (5) years after the last request for the deposit. Should a culture become nonviable or be inadvertently destroyed, or, in the case of plasmid-containing strains, lose its plasmid, it will be replaced with a viable culture(s) of the same taxonomic description.

These deposits are provided merely as a convenience to those of skill in the art, and are not an admission that a deposit is required. The nucleic acid sequences of these plasmids, as well as the amino sequences of the polypeptides encoded thereby, are controlling in the event of any conflict with the description herein. A license may be required to make, use, or sell the deposited materials, and no such license is hereby granted.

Strain	Deposit Date	ATCC No.
pG3HUNEO (in *E. coli*)	9/29/92	69075
pG4HUNEO (in *E. coli*)	9/29/92	69076

Claims

1. An isolated bovine hsp70 promoter capable of directing the transcription of a heterologous coding sequence positioned downstream therefrom.

2. The promoter of claim 1 wherein said promoter is a bovine hsp70A promoter.

3. The promoter of claim 2 wherein said promoter comprises a nucleotide sequence substantially homologous and functionally equivalent to the sequence depicted at nucleotide positions 350 to 441, inclusive, of SEQ ID NO:1.

4. The promoter of claim 2 wherein said promoter comprises a nucleotide sequence substantially homologous and functionally equivalent to the sequence depicted at nucleotide positions 265 to 441, inclusive, of SEQ ID NO:1.

5. The promoter of claim 2 wherein said promoter comprises a nucleotide sequence substantially homologous and functionally equivalent to the sequence depicted at nucleotide positions 1 to 441, inclusive, of SEQ ID NO:1.

6. An isolated bovine hsp70 5′-untranslated region.

7. The 5′-untranslated region of claim 6 wherein said region is a bovine hsp70A 5′-untranslated region.

8. A recombinant expression construct effective in directing the transcription of a selected coding sequence, said expression construct comprising:

(a) bovine hsp70 control sequences according to any of claims 1-7; and

(b) a coding sequence operably linked to said control sequences, whereby said coding sequence can be transcribed and translated in a host cell, and at least one of said control sequences is heterologous to said coding sequence.

9. The recombinant expression construct of claim 8 wherein said bovine hsp70 control sequences comprise a bovine hsp70A promoter and an hsp70 5'-untranslated region.

10. The recombinant expression construct of claims 8 or 9 further comprising an hsp70 3'-untranslated region.

11. The recombinant expression construct of claim 10 wherein said hsp70 3'-untranslated region comprises a human hsp70 3'-untranslated region.

12. The recombinant expression construct of claim 10 wherein said hsp70 3'-untranslated region comprises a *Drosophila* hsp70 3'-untranslated region.

13. The recombinant expression construct of claim 10 wherein said hsp70 3'-untranslated region comprises a bovine hsp70 3'-untranslated region.

14. The recombinant expression construct of any of claims 8-13 wherein said coding sequence encodes

an immunogenic bovine herpesvirus type 1 (BHV-1) gIII polypeptide.

15. The recombinant expression construct of any of claims 8-13 wherein said coding sequence encodes an immunogenic bovine herpesvirus type 1 (BHV-1) gIV polypeptide.

16. The recombinant expression construct of any of claims 8-13 wherein said coding sequence encodes an immunogenic bovine viral diarrhea virus (BVDV) gp53 polypeptide.

17. A recombinant expression construct effective in directing the transcription of a selected coding sequence, said expression construct comprising:

(a) bovine hsp70A control sequences comprising a nucleotide sequence substantially homologous and functionally equivalent to the sequence depicted at nucleotide positions 1 to 666, inclusive, of SEQ ID NO:1; and

(b) a coding sequence operably linked to said control sequences, whereby said coding sequence can be transcribed and translated in a host cell, and at least one of said control sequences is heterologous to said coding sequence.

18. The recombinant expression construct of claim 17, further comprising an hsp70 3'-untranslated region positioned downstream from said coding sequence.

19. The recombinant expression construct of claim 18 wherein said hsp70 3'-untranslated region comprises a human hsp70 3'-untranslated region.

20. The recombinant expression construct of claim 18 wherein said hsp70 3'-untranslated region comprises a *Drosophila* hsp70 3'-untranslated region.

21. The recombinant expression construct of claim 18 wherein said hsp70 3'-untranslated region comprises a bovine hsp70 3'-untranslated region.

22. The recombinant expression construct of any of claims 17-21 wherein said coding sequence encodes an immunogenic bovine herpesvirus type 1 (BHV-1) gIII polypeptide.

23. The recombinant expression construct of any of claims 17-21 wherein said coding sequence encodes an immunogenic bovine herpesvirus type 1 (BHV-1) gIV polypeptide.

24. The recombinant expression construct of any of claims 17-21 wherein said coding sequence encodes an immunogenic bovine viral diarrhea virus (BVDV) gp53 polypeptide.

25. A host cell stably transformed with a recombinant expression construct according to any of claims 8-24.

26. The host cell of claim 25 wherein said cell is a thermotolerant cell.

27. The host cell of claim 26 wherein said thermotolerant cell is a Madin-Darby bovine kidney (MDBK) cell.

28. A method of producing a recombinant polypeptide comprising:

(a) providing a population of host cells according to any of claims 25-27; and

(b) treating said population of cells with heat under conditions whereby said coding sequence is expressed, thereby producing said recombinant polypeptide.

29. The method of claim 28 wherein said recombinant polypeptide is secreted from said host cell.

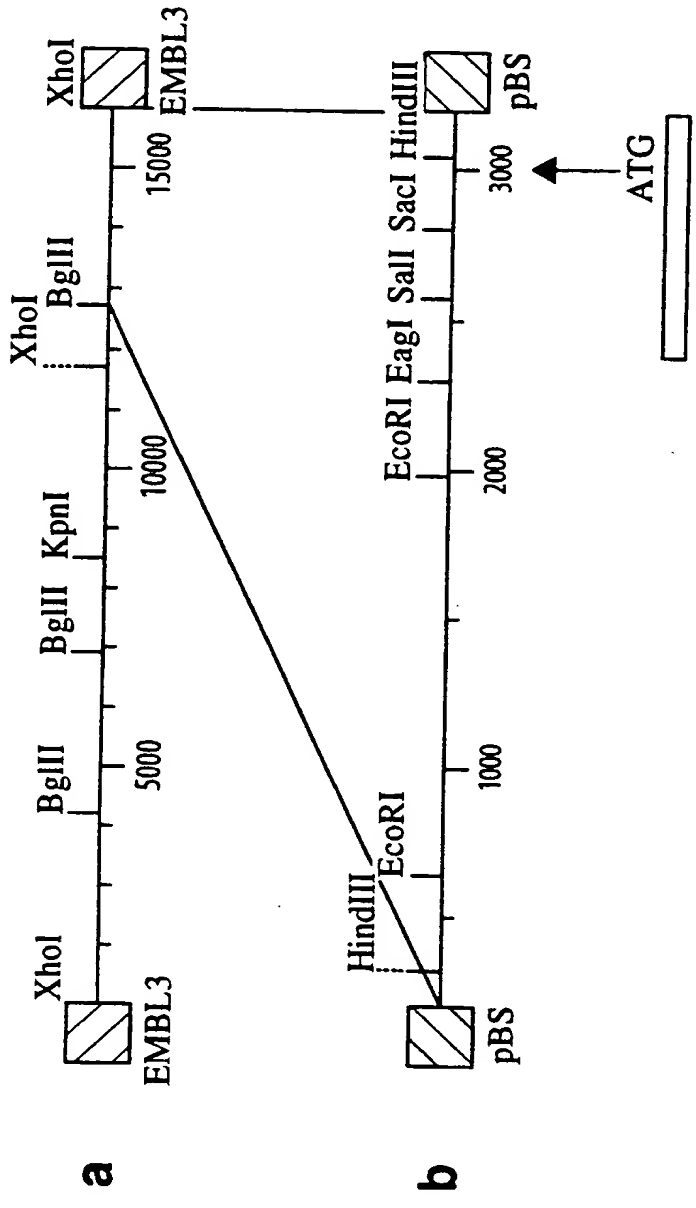

FIG. 1

SUBSTITUTE SHEET

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    TCTTCGAGAAACTCGGGAACTTTCTGTATTTTGGCTGTCCCGGCAGTCGTGTAGC   55
          HSE
    CCTTAATTCTACTTTAAACCACCAAACTAATTTGAGCCCCGAGATCCTCTCACCG  110

    CCCTACAATTAATTACAAGCCCAGGGCTGATCCTTCCAGTCGACTCGACTCCAAA  165

    CTACTTGGCTGGCTGGTCGCCAGGAAACCAGAGACAGAGTGGGTGGACCTTCCCA  220

 40                                                  TTCCT-

    GCCCCTCTCCCCCTCTCCTTAGGACTCCTGTTTCCTCCAGCGAATCCTAGAAGAG  275

 45 --------------CTCA---GGGTCCCTGTCCCCTCCAGTGAATCCCAGAAGAC
                                                     HSE
    TCTGGAGAGTTCTGGGA--GGAGAGGCATCCAGGGCGCTGATTGGTTCCAGAAAG  328

 83 TCTGGAGAGTTCTGAGCAGGGGGCGGCACTCTGGCCTCTGATTGGTCCAAGGAAG
        HSE                                 CCAAT
    CCAGGGGG-CAGGACTTGAGGCGAAACCCCTGGAATATTCCCGACCTGGCAGCCC  382

138 GCTGGGGGGCAGGACGGGAGGCGAAACCCCTGGAATATTCCCGACCTGGCAGCCT
                                  HSE
    CACTGAGCTCGGTCATTGGCTGACGAGGGAAAAGGCGGGGCTTGATGAAGAAT  435

193 CATCGAGCTCGGTGATTGGCTCAGAAGGGAAAAGGCGGGTCTCCGTGACGACT
                  CCAAT      Pu Box  GC
    TATAAACACAGAGCCGCCTGAGGA---GA-AACAGC-AGCCTGGAGAGAGCTGATAA  487

246 TATAAAAGCCCAGGGGCAAGCGGTCCGGATAACGGCTAGCCTGA-GG-AGCTGCTGC

    AACTTACGGCTTAGTCCGT-GAGAGCAGCTTCCGCAGACCCGCTATCTCCAAGGA  541

301 GACAGTCCACTACCTTTTTCGAGAGTGACTCCCGTTGTCCCAAGGCTTCCCAGAG

    CCGCCC---GAGG-----GGCACCAGAGCGTTCAGTTTTCGGGTTCCGAAAAGCC  588

356 CGAACCTGTGCGGCTGCAGGCACCGGCGCGTCGAGTTTCCGGCGTCCGGAAGGAC

    CGAGCTTCTCGTCGCAGATCCTCTTCACCGATTTCAGGTTTGAAGCTTATTTCGG  643

411 CGAGCTC-TTCTCGCGGATCCAGTGTTCCGTTTCCAGCCCCCAATCTCAGAGCCG

    AGCCGGAAAAG--CAGGGCACCGGCATGGCGAAAAACACAGCTATCGGCATCGAC  696

465 AGCCGACAGAGAGCAGGGAACCGC-ATGGCCAAAGCCGCGGCAGTCGGCATCGAC

    CTGGGCACCACCTACTCCTGCGTAGGGGTGTTCCAGCACGGCAAGGTGGAGATC  750

519 CTGGGCACCACCTACTCCTGCGTGGGGGTGTTCCAACACGGCAAGGTGGAGATC
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FIG. 2

SUBSTITUTE SHEET

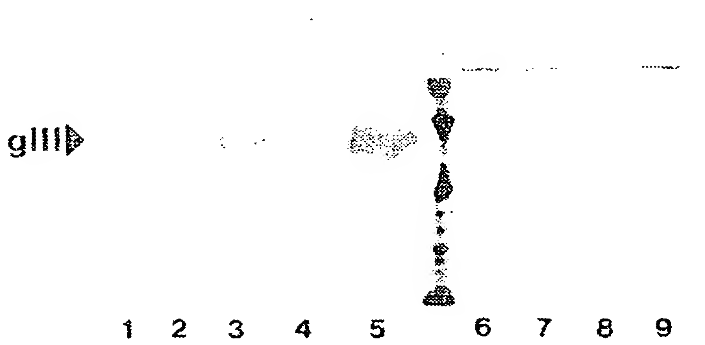


FIG. 3a

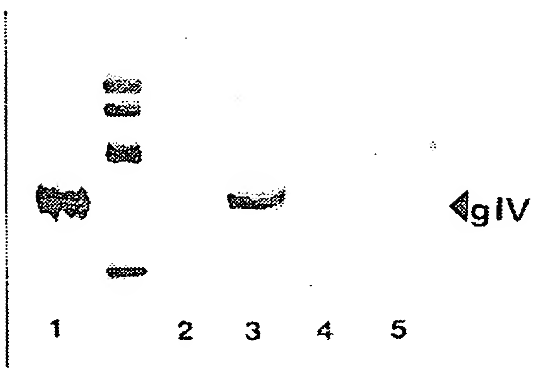


FIG. 3b

FIG. 4a

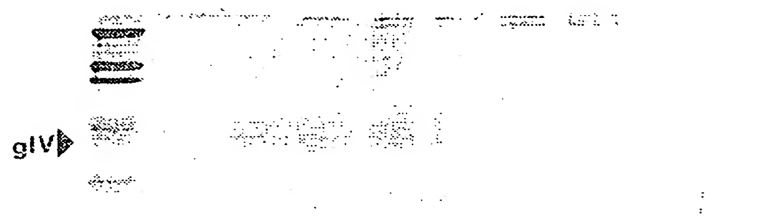
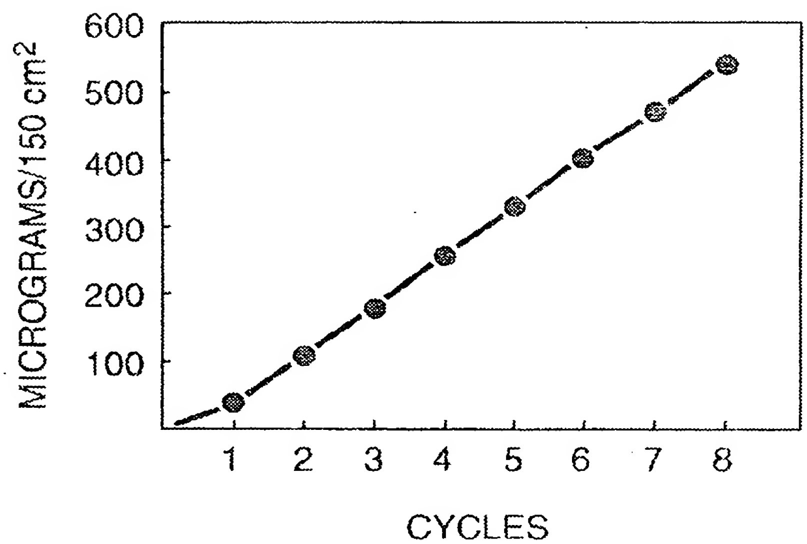

gIV

FIG. 4b


MICROGRAMS/150 cm2
600
500
400
300
200
100
1 2 3 4 5 6 7 8
CYCLES

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 93/00447

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/85 C12N5/10 C12N15/38 A61K39/245 C12N15/40 //C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 118 393 (BATTELLE MEMORIAL INSTITUTE) 12 September 1984 cited in the application see page 12, last paragraph - page 13, last paragraph see page 14, paragraph 3 - page 15, line 6 see claims ---	1,6, 8-12, 17-20
A	US,A,5 151 267 (BABIUK, L. ET AL.) 29 September 1992 cited in the application see column 2, line 45 - line 56 see column 7, line 38 - line 49 see column 10, line 10 - column 12, line 39 see column 18, paragraph II.A.1 --- -/--	14,15, 22,23, 25-29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
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"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
27 January 1994	14. 02. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Andres, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VIROLOGY vol. 165, no. 1 , July 1988 , NEW YORK, US pages 191 - 199 COLLETT, M. ET AL. 'Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus' cited in the application see the whole document	16,24
A	WO,A,87 00861 (BATTELLE MEMORIAL INSTITUTE) 12 February 1987 cited in the application see the whole document	1
P,X	VACCINE vol. 11, no. 11 , August 1993 , GUILDFORD GB pages 1100 - 1107 KOWALSKI, J. ET AL. 'Heat-shock promoter-driven synthesis of secreted bovine herpesvirus glycoproteins in transfected cells' see the whole document	1-10,13, 14,17, 18, 21-23, 25-29

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern   ıl Application No
PCT/CA 93/00447

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		AU-A- 2420884	17-04-86
		CA-A- 1312027	29-12-92
		JP-A- 59192091	31-10-84
US-A-5151267	29-09-92	NONE	
WO-A-8700861	12-02-87	AU-B- 604214	13-12-90
		AU-A- 6286086	05-03-87
		EP-A- 0231368	12-08-87